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Upon the intracellular constituents of the typhoid bacillus.

[From the Jenner Institute of Preventive Medicine, London.]

By Dr. **Allan Macfadyen** and **Sydney Rowland.**

With 2 Figures.

The following paper contains an account of the results that have been obtained as regards the typhoid bacillus since the publication of our first communication in the Centralblatt für Bakteriologie. Abt. I. Vol. XXX. 1901. No. 20.

The investigations undertaken had, as was then stated, a special object in view, viz: the study of certain of the intracellular factors in health and disease by obtaining directly the cell constituents and eliminating as far as possible excreted substances and those formed by the cell in a given environment. The ordinary laboratory methods could not be employed for this purpose, and it was necessary in the first instance to devise the means of carrying out the research. The progress of the inquiry has therefore necessarily been slow, as many technical difficulties had to be overcome. The investigation has now been successfully advanced in various directions. The intracellular juices of healthy and morbid tissues, of leucocytes and of a number of micro-organisms have been obtained and submitted to examination by the writers and their colleagues. The results, in so far as published, are referred to at the end of the paper. The experiments carried out with the typhoid organism and the results obtained were of the following nature.

I. Experiments with reference to an extracellular typhoid toxin.

The existence of a specific toxin produced by the typhoid bacillus has hitherto not been demonstrated although it has been assumed by analogy with other organisms, and by reasoning from the clinical course of the disease. Such a poison must be either extracellular or intracellular. The endeavours however, to demonstrate the production of an extracellular toxin by the typhoid bacillus have not hitherto led to any definite results. That a toxin of this character does not exist in filtered cultures of the organism is the common experience of bacteriologists. The typhoid organism when grown in the ordinary culture media does not produce any soluble products with marked poisonous properties. The absence from such cultures of definite toxins might be due to the unsuitability of the soil used for growing the typhoid bacillus.

We considered it of importance to retest the question, since the detection of such a toxin would constitute a great advance in the understanding and the treatment of the disease.

The first step in the search for the body in question consisted in substituting for the usual broth and peptone media, culture fluids approaching more nearly in constitution the natural body soils which clinically support the life of the bacillus. A number of experiments extending over a year were made in this direction. We endeavoured to cultivate the typhoid bacillus in fresh juices obtained from various organs of the

animal body, and representing the intracellular juices of the tissues. The spleen, lymphatic glands and intestinal mucous membrane were triturated and the juices expressed at a low temperature in order to prevent changes in the material during the grinding process. Such media would approximate more closely in constitution to the substances the typhoid organism might be expected to meet in the course of its stay in the body of a host in which it is producing toxic symptoms, and particularly if heat were avoided in their preparation. The fresh organs or tissues from the ox or the calf, as received from the slaughterhouse, were finely minced in a mincing machine and the resulting pulp disintegrated according to the methods employed by us in the preparation of Zymase, viz: with the aid of sand, the mass being kept cool during the process by an outer jacket of brine or carbonic acid (1).

The fresh juices thus obtained were passed through a Berkefeld filter to ensure sterility, and in each instance the intracellular juice was brought to the requisite degree of alkalinity by the addition of sodic carbonate. These juices were employed as a culture soil for the typhoid bacillus under the following conditions:

1) The organ juice per se.

2) The organ juice with an admixture of fresh human serum.

3) The organ juice after heating to 55° C for twenty minutes, and with or without the subsequent addition of fresh human serum.

The above conditions were applied to organ juices obtained from the fresh spleen of the ox, the lymphatic glands (mesenteric) of the calf and the intestinal epithelium of the ox and a few other animals. The media were inoculated with the typhoid bacillus alone and in a series of experiments, with the typhoid bacillus in conjunction with the bacillus coli communis. In every instance a parallel series of cultures was made under aerobic and anaerobic conditions. We were able to cultivate the typhoid bacillus under the above conditions and to determine in how far its toxicity was thereby affected. After an incubation at blood heat for four weeks the cultures were examined as to the presence of growth and freedom from contamination. The cultures were then passed through a Berkefeld filter to remove the organisms, and the filtrates injected into animals in small and large doses (e. g. 5 cub. c. and more). The experimental animals were guinea pigs, rabbits and monkeys. With the possible exception of one spleen juice, none of the fluids thus obtained exhibited any acute toxic power, either when used as culture soils for the typhoid organism or in conjunction with the colon bacillus. In the case of the rabbit and the monkey the fluids were practically innocuous. As regards the guinea pig no immediate toxic effect was observed. In a certain number of cases, however, the guinea pigs eventually died at the end of a period which averaged about six weeks. If one excludes the possibility of substances toxic to the guinea pig being naturally present in the organ juices (of which we possess a certain amount of experimental evidence), the result might be interpreted as being due to some slowly acting soluble toxin or toxins derived from the typhoid bacillus. We have not however as yet been able to observe any distinct effects on post mortem examination, and are at present unable to ascribe any definite significance to the result. It will be sufficient in the meanwhile to record the fact, and to omit the table of results as they are not essential to the present paper. Such toxins, if they exist, are quite different in properties to the intracellular toxin we are about to describe. The experiments were suf-

ficient to lead us to the conclusion that in no case was an extracellular toxin developed comparable in any way to those obtained from pure cultures of undoubtely extracellular toxin-producing organisms, e. g. the diphtheria bacillus, etc. It did not appear that this line of investigation would be likely to lead to any practical results. The very large number of experiments made with the most natural soils obtainable had not been successful in demonstrating the presence in cultures of the typhoid bacillus of any definite toxin of likely value for immunising purposes.

II. Experiments with reference to an intracellular typhoid toxin.

The experiments having failed to establish the presence of any definite extracellular toxin, it became necessary to search within the typhoid organism itself for the missing toxin. The research was thus directed not to the products of the typhoid bacillus, but to the organism itself and its intracellular constituents. For this purpose the endeavour was made to obtain the fresh unmodified cell plasma of the organism and the method originally employed was as follows: The virulent typhoid bacilli were grown on the surface of nutrient agar in flat rectangular bottles, each giving a surface of 200 sq. cm; one hundred such culture bottles were required in order to yield a growth sufficient for trituration by the method that was in the first instance adopted. After cultivation for about 36 hours at blood heat, the bottles on being washed out with salt solution, yielded about one litre of a thick emulsion of the bacilli. The bacilli were separated from the emulsion by means of a high speed centrifuge, and were at the same time thoroughly washed free of possible excretory products by repeated additions of physiological salt solution. The washed and separated bacilli were then mixed with fine silver sand and triturated in a cold-jacketed metal cylinder by means of small vanes revolving at a high velocity. The intercollision of sand particles and bacilli resulted in the rupture of the bacterial cells, and the process usually occupied from three to four hours. The resultant mass was filtered through Kieselguhr with the aid of a hydraulic press. The filtrate represented a rich watery solution or suspension of the intracellular constituents of the typhoid bacillus in so far as these were capable of passage through the Kieselguhr. There remained at the end of the pressing a hard cake of Kieselguhr, which was found to contain a considerable amount of retained albuminous and other organic substances. Repeated extractions of this cake, made with glycerine and with a solution of carbonate of soda, demonstrated that the Kieselguhr cake contained physiologically active constituents of the typhoid organism. There had undoubtedly been held back intracellular elements of possible importance to the experiments we desired to carry out. The entire operation lasted about six hours and the average yield of juice from the first pressing was about 8 ccm. An account has already been given of the experiments made with such juices upon guinea pigs and rabbits with a view to testing their toxicity and immunising properties against the bacillus typhosus. It was found that the fluid, injected in doses of 1, 0.5 and 0.2 ccm completely protected the experimental animals against one to ten lethal doses of virulent typhoid bacilli, and the protection following one such injection lasted about four weeks. The results were identical whether a first, second or third pressing of the juice through the Kieselguhr was employed. The juices preserved their immunising properties as regards the typhoid bacillus for a considerable period of time,

as at the end of four months they were still found to be active in this respect. The cellplasma on subcutaneous inoculation was very quickly absorbed without evidence of local irritation. The quick absorption of the cell juices by the tissues and the absence of local irritation we regard as a point of considerable practical importance. If the full immunising effect as regards the typhoid bacillus per se is to be attained by the injection of the plasma obtained from its cell substance, such a method of procedure would undoubtedly present considerable advantages over the other methods that have hitherto been employed with the same end in view, e. g. the use of heated cultures and the intact bodies of the bacilli as vaccines etc. The ideal method of procedure would be to obtain an immunising substance directly from the bacterial cells, of nonirritating properties, capable of rapid and complete absorption by the tissues, and freed from all the superfluous material present in the ordinary culture media.

In this respect the methods we were employing appeared to furnish the hope of obtaining an active and at the same time a purer material than had hitherto been found possible. The appearance of the agglutination reaction in the blood of the treated animals afforded evidence that we were dealing with intracellular juices which possessed active physiological properties. This reaction appeared very quickly and persisted for a considerable period of time, and was still present when the specific protective substances had disappeared from or ceased to be active in the blood. In the case of the rabbit an agglutination of the typhoid bacillus occurred nine months subsequent to the injection of the typhoid cell juice subcutaneously. On intravenous injection we have succeeded in obtaining the agglutination reaction within seventeen hours, and at times in two hours, after inoculation with a dilution of 1 in 100 of rabbits blood. One injection of the cell juice was sufficient to develop antibacterial properties in the blood of the treated animals. At the end of a month the serum was actively bacteriolytic. A complete destruction of the typhoid bacilli by the serum in doses of $^1/_{10}$, $^1/_{20}$ and $^1/_{50}$ ccm, occured within two hours. The agglutinative and bacteriolytic action was obtained with the blood serum of treated rabbits and monkeys.

The experiments at this stage had demonstrated that the typhoid cell plasma, obtained by the above methods, possessed active physiological properties, and that on injection they afforded a certain protection against virulent typhoid organisms in virtue of specific bacteriolytic properties developed in the blood of the treated animals. At the same time the yield of active cell plasma by the above mentioned triturating process did not prove to be of a quantitative character. A considerable amount of the cell constituents was retained in the Kieselguhr sponge. The method appeared in this respect to be capable of improvement and particularly with reference to the minute cells that we were dealing with. A method which would eliminate the sand and Kieselguhr, as employed by other observers (Buchner, Hahn etc.) and by ourselves, and would at the same time produce a rapid trituration of the organisms was, we found by experience, essential. The method, if it could be successfully devised, would yield the entire intracellular constituents of the micro-organisms in question for the purpose of experiment. We had likewise noted the tolerance exhibited by the treated animals, and particularly by the guinea pig, to the injection of large quantities of the expressed cell plasma of the typhoid bacillus. Whilst the immunising properties of the cell juices, as

regards the typhoid organism, had been demonstrated, acute and definite toxic effects had proved remarkable by their absence.

These various observations led us to endeavour to improve the methods employed, and to relinquish the procedure on lines analogous to those of Buchner, Hahn and other investigators in the study of expressed cell juices. The results, on the injection of such expressed cell juices into animals, were purely of an antibacterial character, an active toxin in the cell plasma and consequently antitoxic properties in the blood of the treated animals had not been demonstrated. This constituted a serious gap in the experiments, if we assume that an intoxication of the system in the case of enteric fever is a grave and perhaps the cardinal factor to be considered in the treatment of the disease.

The filtering action of the Kieselguhr used in the filter pressing appeared to be the most likely reason for lack of success in this direction. The disintegration of the organisms was therefore attempted without the admixture of any foreign material which would render a subsequent filtration through Kieselguhr necessary.

III. Experiments with cold grinding methods.

The mechanical method of disintegration that appeared to be most likely to lead to successful results in the case of bacteria was their trituration whilst in a frozen and brittle condition. It had already been demonstrated (2) that an exposure to the temperature of liquid air (about —190° C) did not injure or destroy the vitality of bacteria, and that micro-organisms might be kept for as long a period as six months at this low temperature without any deleterious effect.

This important point being determined, it appeared probable that the brittleness of the cells at this low temperature would favour their mechanical disintegration without any admixture of sand or other foreign substance. The most convenient agent for the production of the necessary cold was liquid air. Liquid air possessed two practical advantages: — it could be more conveniently handled than other substances that might possibly have given the necessary conditions of cold at higher temperatures than — 190° C and it furnished a fluid freezing bath in which the material to be ground could be directly immersed. These properties have proved of great practical value in the course of the experiments. A further advantage was that at such a temperature the ordinary chemical processes would cease, changes due to heat would be eliminated, and the process if successful would furnish a quantitative yield of unaltered cell plasma.

The experiments were successful and the feasibility of disintegrating micro-organisms per se, without any admixture of triturating substances was demonstrated. The complete disintegration of the typhoid bacillus was accomplished at the temperature of liquid air in a period of about two hours without the addition of sand or other foreign substance.

The method has likewise been successfully applied to a number of bacteria, to other types of vegetable cells, and to animal organs and tissues, and their intracellular juices obtained for experimental purposes.

The method entirely obviates the use of any accessory grinding or filtering substances and fulfils the conditions we desired to obtain for the study of intracellular constituents.

These conditions were as follows:

1) That no chemical or heat changes should take place during the process of disintegration,

2) That the disintegration should be accomplished without the addition of any triturating substance, the necessary subsequent removal of which might vitiate the composition of the resulting mass.

3) That the process should furnish a quantitative yield of the unmodified cell plasma.

In this communication we will confine ourselves to the results obtained with the typhoid bacillus. (Schluß folgt.)

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With 2 Figures.

(Schluß.)

IV. Apparatus and methods.

It will be advisable in the first instance to give a full description of the methods that have been specially devised and employed for obtaining directly the intracellular juices of the typhoid bacillus and other organisms. The general principle consists in freezing the micro-organisms to an extreme degree of brittleness by means of liquid air, and disintegrating the cells per se in a mechanically operated mill.

In the case of the expressed juices obtained by the sand and Kieselguhr method, about 100 agar culture bottles were required to furnish an adequate growth of the organisms for grinding purposes. In the present method, ten such agar cultures are sufficient for a single grind of the micro-organism in question. This in itself is a great saving in time and material.

The virulent typhoid organisms are grown on the surface of ten agar bottles at blood heat for 24 to 30 hours. The growth is then washed off with salt solution and the resultant emulsion of bacilli is spun in a high speed centrifuge. The process is repeated several times with freshly added salt solution, in order to cleanse the organisms from any extraneous matter. The spun out bacteria are next reduced to the consistency of a pasty mass by a rapid drying on the surface of a Chamberland filter through which air is being sucked.

The average yield of washed bacteria, when freed as far as possible from adherent water, was about 0.15 g per culture plate. This represented quantitatively 1.5 ccm

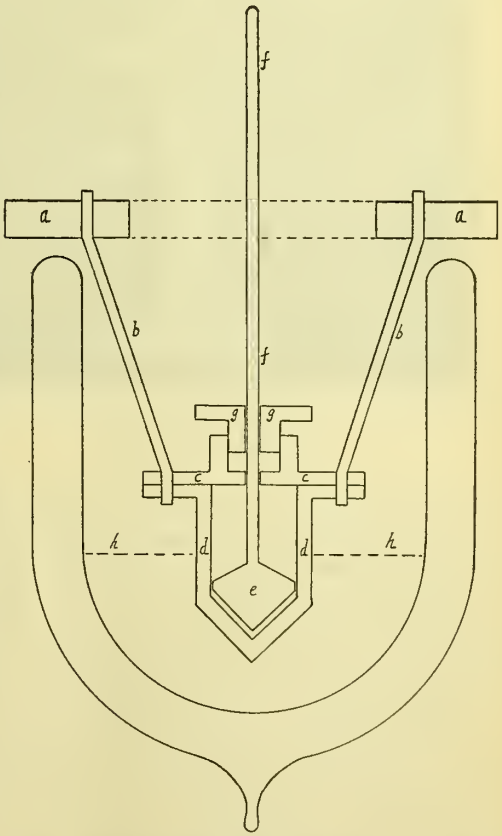

Fig. 1. Diagrammatic vertical section of liquid air grinding apparatus.

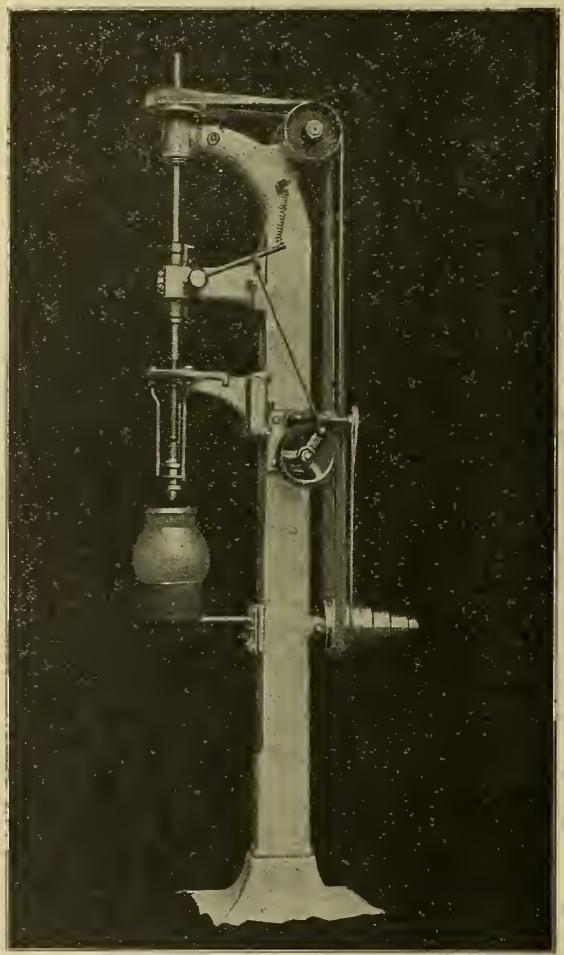

Fig. 2. Photographic reproduction of liquid air grinding apparatus.

of the 10 per cent salt solution of the cell juice prepared after the disintegration of the cells.

The pasty mass of washed organisms is removed from the Chamberland filter and is introduced into the apparatus shown in diagrammatic section in fig. 1. This apparatus is constructed as follows: The horizontal plate *A* (also seen in the photographic reproduction fig. 2) supports by the rods *BB* the plate *CC*. This plate, circular in plan forms the cover of the conical receptacle *DD* on which is free to revolve the doubly coned plunger *E*. The vessel *D* can be removed from the covering plate *C*, to which it is attached by bolts, for the purpose of introducing the material to be disintegrated, as in this instance the typhoid bacilli. The plate *CC* is furnished with a gland *GG*, closely packed with ignited asbestos, through which works the rod *F*, which rigidly supports the cone *E*. The joint between *CC* and *DD* is made by means of an annular paper washer. A continuous rotary motion is imparted to *F* by means of the mechanism shown in fig. 2. At the same time a reciprocating motion is imparted by the worm and wheel gear seen at the side of the main supporting columns of the apparatus. When in operation, the apparatus is immersed in liquid air up to the level *HH*.

The operation of the machine is as follows. On the descent of *F*, *E* is kept rotating with considerable pressure against *D*, and the surface of *E* being finely knurled, any material between *E* and *D* is reduced to powder, in which condition it finds its way into the upper cone of *E*. On the ascent of *F* (still revolving) this coarse powder falls to the bottom of *D*, and is again forcibly rubbed between the lower cone of *E* and the bottom of *D*, on the next descent of *F*. This sequence of operations is

continued until no entire micro-organisms are found on microscopical examination, a result which can be accomplished in from $1^1/_2$ to 2 hours, when dealing with 0.5 to 1 g of pasty organisms. It must be remembered that as the above sequence of operations will take place at about — 190° C., the contents of *D* are in the condition of a dry powder.

As a result of this process, there is obtained a pasty mass (when thawed), consisting of the entire substance of the organisms used. Moreover, this substance, at the moment of thawing, is chemically identical with the living protoplasm of the cell, the whole disintegration having occurred under conditions which preclude the possibility of any chemical change taking place.

This pasty mass is mixed with salt solution (0.75 p. c.), rubbed up in an agate mortar, and the opaque suspension thus obtained is centrifugalised until free from suspended matter, and an opalescent solution of the intracellular constituents of the organism results.

The centrifugalisation of such minutely divided material is occassionally a matter of difficulty, more especially if, by chance any unground organisms are found in the suspension. In those cases, in which a sterile juice is required the centrifugalisation must be very vigorous, and for this purpose it has been found that the best results are given by a very simple form of centrifuge, consisting of a horizontally mounted steel disc (36 inches in diameter) on the periphery of which are mounted strong glass tubes in steel cases hung on trunnions, the disc making from 3,000 revolutions per minute. The glass tubes to prevent fracture under the great strain are floated in mercury, contained in the covering steel tubes. With such a disc, sterile juices have been obtained.

The centrifugalised celljuice was as a rule equivalent to a 10 per cent solution of the intracellular constituents obtained. It represented the intracellular constituents of the typhoid organisms soluble in physiological salt solution, after a complete disintegration of the cells had been accomplished.

It is this material which was used in the experiments we are about to describe and it consisted of a bacterial celljuice practically identical with that contained within the living cell.

These juices likewise had the merit of possessing a constant constitution, and thus were capable of being standardised as regards their physiological action.

V. Demonstration of intracellular toxin.

The first experiments were carried out with the view of testing whether the typhoid celljuices obtained by cold grinding methods possessed any toxic properties. It was found that if such a disintegrated mass be freed from whole bacilli (if present) and from other suspended insoluble particles by centrifugalisation, an opalescent fluid results which on inoculation into animals in small doses, invariably proves toxic or fatal. It was therefore concluded that the typhoid bacillus contains within itself an intracellular toxin. The toxin thus obtained and employed for experiment is a ten per cent solution in normal saline of such intracellular constituents of the typhoid bacillus as are soluble in such a medium.

The standard adopted in estimating the toxicity of the typhoid celljuice was the amount found to prove fatal on intraperitoneal injection into the guinea pig. The intracellular fluid under these circumstances invariably proved toxic in a short period of time. The toxicity of the juices

varied pari passu with the virulence of the living bacilli for the guinea pig. The greater the virulence of the organism employed, the greater was the toxicity of the intracellular constituents obtained from it, and vice versa. For example, the toxicity of the plasma obtained from an organism of which 0.1 ccm of a broth culture killed in a few hours was greater than in the case of an organism of which larger quantities of a broth culture failed to produce death in the same period of time.

In the case of a broth culture of the typhoid organism of such a degree of virulence that $^1/_{10}$ of a cube on intraperitoneal injection, produced death infive to ten hours, the toxicity of the celljuice obtained from the same organism would be on an average as follows. On intraperitoneal injection, 1 ccm of such a toxin killed in 3 hours; 0.5 ccm in 3 to 4 hours; 0.2 and 0.1 ccm in 3 to 5 hours; 0.05 ccm in about 12 hours and 0.02 ccm in about 40 hours.

We have likewise obtained a juice of which 0.02 ccm has killed in six hours.

The best result so far obtained as regards acute toxicity, was in the case of a 10 per cent toxin of which 0.003 ccm killed within 24 hours.

No organisms were found in the blood or peritoneal cavity on postmortem examination. The effects were therefore produced by "devitalised" constituents of the typhoid bacillus. The peritoneal cavity contained a considerable amount of exudation; haemorrhages were present in the stomach; the small intestine was acutely congested and the suprarenal capsules were injected.

Similar results to the above were obtained in a very large number of repetition experiments and justified the conclusion that the typhoid bacillus contains an intracellular soluble toxin of considerable power.

The toxin on subcutaneous injection into the guinea pig produced a toxin oedema at the seat of inoculation, and death has occurred after the injection of 0.5 ccm and 0.2 ccm of the toxin in about seven days.

One of the effects of a sublethal dose of the toxic juice was the early and constant appearance in the blood of marked agglutinating properties, and sometimes this occurred a few hours after e. g., an intravenous injection. The constant presence of this reaction served to demonstrate the specificity of the celljuices with which we were dealing.

The heat relationships of the toxins derived from the typhoid bacillus and other organisms are being investigated, and the results will be published in due course.

VI. Immunising properties of the typhoid celljuices.

It remained to test the typhoid celljuices for immunising and other properties. The preliminary experiments in this direction were made upon the rabbit and the monkey. The monkey was selected as an animal most likely to furnish data of possible application to man. For this purpose the typhoid celljuice was administered subcutaneously to the monkey. The injections did not produce any general symptoms beyond a transient rise in temperature, whilst the material was quickly absorbed after each injection without any traceable effect.

In this manner doses of 0.5 to 1 ccm of the material were injected at intervals. An immediate result was the agglutination of the typhoid bacillus by the serum of the treated monkeys, whereas no such effect was produced by the serum of monkeys which had not been treated.

This furnished useful evidence that the animals were under the in-

fluence of celljuices derived from the typhoid organism. The injections were repeated at intervals of 3—4 days, and after a lapse of 4—6 weeks the animals were bled.

The serum obtained was then tested for immunising properties. The test objects were 1) a virulent culture of the typhoid bacillus and 2) the intra-cellular toxic juice of the same organism. A varying amount of the virulent bacilli and of their toxic celljuice was mixed with a varying quantity of the serum. The respective mixtures were then injected into the peritoneal cavity of the guinea pig.

The broth cultures of the typhoid organism used in the experiments were per se lethal in doses of 0.1 ccm in 5—10 hours. The typhoid celljuices were fatal in doses of 0.2 and 0.1 ccm in 3—5 hours and in doses of 0.05 ccm in about 12 hours. The serum was thus tested for 1) specific antibacterial and 2) specific antitoxic properties.

The experiments showed that the serum of the monkey, after injection of the typhoid celljuices, possessed antibacterial and antitoxic properties, inasmuch as the serum protected the experimental animals against the bacilli, and also against an intracellular toxin obtained from them.

A simultaneous injection of 1) serum with the bacilli, and 2) serum with the toxic celljuice produced no lethal or toxic effects. The control animals on the other hand invariably succumbed.

It was further investigated whether the serum possessed preventive and curative properties.

The serum from treated monkeys was injected into guinea pigs, one injection being made in each instance, and the same animals received at an interval of 12—24 hours lethal doses of the typhoid bacillus and of its toxic intracellular juice respectively. The treated animals survived the test, whilst the control animals succumbed. It was therefore concluded that the serum had protective properties.

A third series of guinea pigs received lethal doses of the typhoid bacillus and of its toxic celljuice respectively. The serum was then injected at various intervals into individual animals. It was found that the lives of the animals could be saved by one injection of the serum, from a fatal infection or intoxication, even when half of the lethal period had elapsed in each instance. The serum therefore, possessed curative properties. From the experiments made upon the monkey it would appear 1) That by the injection of the intracellular juices of the typhoid organism into the monkey, it is possible to obtain a serum with both antibacterial and antitoxic properties; 2) That such a serum possesses curative and preventive properties as regards the typhoid bacillus and an intracellular toxin present in the same organism. It is believed that this has furnished for the first time proof that in the case of one species of pathogenic organism, the intracellular juices of the organism when injected into a suitable animal, give rise to the production of a serum which is both bactericidal to the organism itself and antitoxic as regards a toxin contained in its substance. How far such properties of the celljuice are shared by other pathogenic organisms is being made the subject of further inquiry.

In the case of the rabbits treated with the typhoid celljuice, antibacterial and antitoxic properties were likewise found to be developed in their blood. The experiments which have been made with the goat are confirmatory of the above results, its serum likewise possessed antibacterial

and antitoxic properties as regards the typhoid bacillus and the soluble toxin derived from it. At the present moment the experiments are being conducted on the horse.

The in vitro experiments that have been made with the various serums obtained have confirmed the results obtained in the experimental animals.

In was important to determine, whether in addition to being antitoxic, the serum obtained from the experimental animals was likely under further treatment to possess an enhanced antitoxic value, and whether as in the case of diphtheria, any "overproduction" of antitoxin could be demonstrated. We have found that the serum of an animal immunised by repeated injections of celljuice in doses of 0.2 ccm, can completely neutralise thetoxic effect of one hundred times the amount of a typhoid celljuice that is capable of producing death on intraperitoneal injection into the guinea pig in six hours.

We conclude, therefore, that as the antitoxic value can be raised by repeated injections, there is every reason to hope that it can be still further raised by longer treatment.

To those familiar with Ehrlich's elaborate standardising methods with regard to the diphtheria toxin, the absence of similar data from this paper will no doubt be noted. It will, however, be equally obvious that a method of standardising and testing which has after much experiment by many observers, reached a high empirical standard of efficiency cannot be applied to a toxin of an entirely different nature.

We are engaged in the consideration of the best method of standardisation to be adopted with special reference to the typhoid toxin and intracellular toxins in general, and the lengthy experiments involved have not yet been completed. We must therefore, content ourselves with giving the protocoll of a typical experiment.

Experiments with serum from monkey B.

Injections intraperitoneal.

0.2 ccm toxin killed in 4½ hours.
0.25 „ broth cult. killed in 10 hours.

A. Injection of serum followed by injection of typhoid culture and toxin.

At 5 p. m. injection of serum made.

Guinea Pig 1	Guinea Pig 2	Guinea Pig 3	Guinea Pig 4
0.5 ccm	0.25 ccm	1 ccm	1 ccm

the following day at noon

0.25 typ. cult.	0.25 typ. cult.	0.2 toxin	0.1 toxin

B. Simultaneous injection of serum aud broth culture of typhoid bacillus.

Guinea Pig 5	Guinea Pig 6	Guinea Pig 7	Guinea Pig 8
0.5 serum	0.25 serum	0.1 serum	0.05 serum
0.25 broth cult.	0.25 broth cult.	0.25 broth cult.	0.25 broth cult.

C. Simultaneous injection of serum and toxin.

Guinea Pig 9	Guinea Pig 10	Guinea Pig 11	Guinca Pig 12
1.0 ccm serum	0.5 ccm serum	0.2 ccm serum	0.1 ccm serum
0.2 „ toxin	0.2 „ toxin	0.2 „ toxin	0.2 „ toxin

D. Injection of typhoid bacillus followed by injection of serum.

Guinea Pig 13	Guinea Pig 14	Guinea Pig 15
At 12.50 0.25 ccm typ. broth cult.	0.25 ccm typ. broth cult.	0.25 ccm typ. broth cult.
At 1.30	At 2.30	At 3.30
0.25 ccm serum	0.4 ccm serum	0.5 ccm serum

Guinea Pig 16	Guinea Pig 17
At 12.50 0.25 ccm typ. broth cult.	0.25 ccm typ. broth cult.
At 4.30	At 5.30
0.4 ccm serum	0.5 ccm serum

E. Injection of toxin followed by injection of serum.

Guinea Pig 18	Guinea Pig 19	Guinea Pig 20	Guinea Pig 21	Guinea Pig 22
At 1 p. m.	At 1 p. m.	At 1 p. m.	At 1 p. m.	At 1 p. m.
0.2 ccm toxin	0.2 ccm toxin	0.2 ccm toxin	0.2 ccm toxin	0.2 ccm toxin
At 1.30 p. m.	At 2 p. m.	At 2.30 p. m.	At 3 p. m.	At 3.30 p. m.
0.2 ccm serum	0.5 ccm serum	0.7 ccm serum	1 ccm serum	1 ccm serum

All the animals survived the above test with the exception of No. 2 which died after two days, and No. 21 which survived $4^1/_2$ hours.

VII. General conclusions.

Experiments are at present being conducted on the horse.

It remains to be seen in how far the results already obtained are capable of being utilised outside the laboratory in clinical directions.

It appears to us that the results detailed above possess considerable theoretical interest. The experiments have furnished a demonstration of the fact that it is possible to prepare a serum in the case of a given organism which is bactericidal to the organism in question and antitoxic as regards a toxin contained within its substance. Further, the experiments by the demonstration of the presence of a specific intracellular toxin, may, it is possible, serve to explain the most striking feature in the course of typhoid fever — the intoxication.

As regards the practical methods of preparing bacteriolytic serums, an immunisation of the animals by means of disintegrated cells offers many advantages in practice, through the absence of serious local reaction and the rapidity of absorption of the inoculated material.

There appears also, the possibility of obtaining bacterial vaccines of greater purity and capable of more accurate standardisation in the case of Enteric Fever, of Plague and other diseases, the symptoms of which may depend upon the presence of intracellular toxins in their exciting organisms. This matter is one that is engaging our careful attention.

In conclusion we have to express our appreciation of the valuable advice and help afforded by Professor James Dewar. F. R. S. in the course of these and other investigations.

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